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INTEGRATIVE PHYSIOLOGY

Regulation of mineral metabolism by lithium

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Abstract Lithium, an inhibitor of glycogen synthase kinase 3 (GSK3), is widely used for the treatment of mood disorders. Side effects of lithium include nephrogenic diabetes insipidus, leading to renal water loss. Dehydration has in turn been shown to downregulate Klotho, which is required as co-receptor for the downregulation of $1,25(OH)_2D_3$ formation by fibroblast growth factor 23 (FGF23). FGF23 decreases and $1,25(OH)_2D_3$ stimulates renal tubular phosphate reabsorption. The present study explored whether lithium influences renal Klotho expression, FGF23 serum levels, $1,25(OH)_2D_3$ formation, and renal phosphate excretion. To this end, mice were analyzed after a 14-day period of sham treatment or of treatment with lithium (200 mg/kg/day subcutaneously). Serum antidiuretic hormone (ADH), FGF23, and $1,25(OH)_2D_3$ concentrations were determined by ELISA or EIA, renal Klotho protein abundance and GSK3 phosphorylation were analyzed by Western blotting, and serum phosphate and calcium concentration by photometry. Lithium treatment significantly increased renal GSK3 phosphorylation, enhanced serum ADH and FGF23 concentrations, downregulated renal Klotho expression, stimulated renal calcium and phosphate excretion, and decreased serum $1,25(OH)_2D_3$ and phosphate concentrations. In conclusion, lithium treatment upregulates FGF23 formation, an effect paralleled by substantial decrease of serum $1,25(OH)_2D_3$, and phosphate concentrations and thus possibly affecting tissue calcification.

Keywords Lithium · FGF23 · Klotho · $1,25(OH)_2D_3$ · Phosphate · Calcium

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Introduction

Fibroblast growth factor 23 (FGF23), a hormone released mainly from osteoblasts [25, 47], is a powerful regulator of calcium phosphate metabolism [23, 51]. As previously shown [3, 4, 42, 51, 69, 71, 73, 75], FGF23 inhibits renal tubular phosphate reabsorption. Moreover, FGF23 downregulates renal 1α hydroxylase (Cyp27b1) and upregulates 25-hydroxyvitamin D 24-hydroxylase (Cyp24), effects leading to decreased formation and increased catabolism of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) [18, 26, 72, 75]. FGF23, thus, lowers serum $1,25(OH)_2D_3$ levels [3, 4, 26, 62, 69, 71, 73, 75]. $1,25(OH)_2D_3$ is a powerful regulator of renal and intestinal phosphate and calcium transport [10, 54, 55, 68]. FGF23 increases renal elimination of phosphate [3, 4, 69, 71, 73, 75]. FGF23 deficiency elevates serum phosphate, calcium, and $1,25(OH)_2D_3$ levels, effects eventually leading to several severe disorders, such as vascular calcification, decrease of bone

density, growth retardation, infertility, and a dramatic reduction of lifespan [15, 49, 67, 71, 72, 76].

To become effective, FGF23 requires Klotho as co-receptor [25, 36, 37]. Accordingly, mice with reduced Klotho expression similarly suffer from multiple age-related disorders with growth retardation, extensive soft tissue calcification, and decreased life span paralleled by osteopenia/osteoporosis, endothelial dysfunction, impaired angiogenesis, sinoatrial node dysfunction with sudden cardiac arrest, enhanced erythrocyte turnover, pulmonary emphysema, skin atrophy, hypogonadotropic hypogonadism, infertility, muscle dystrophy, hearing loss, neuron degeneration, Parkinson's disease, cognition impairment, neoplasms, inflammation, and tissue fibrosis [5, 11, 17, 29, 35, 37–39, 50, 56, 57, 63, 74, 79, 82, 83]. Klotho deficiency is largely effective through strong increases in $1,25(OH)_2D_3$ formation, inhibition of renal tubular phosphate transport, and subsequent elevation of serum phosphate levels [14, 24, 87]. Hyperphosphatemia predisposes to vascular calcification [20] and is recognized as predictor of mortality [81].

Recent observations revealed that renal Klotho expression is markedly downregulated by dehydration [80]. Drugs causing dehydration include lithium [21, 30], which is widely used in the treatment of bipolar disorders and Alzheimer's disease [16, 28, 52]. Lithium treatment interferes with renal effects of antidiuretic hormone (ADH), thus causing nephrogenic diabetes insipidus [8, 31, 43, 65, 77]. Lithium is at least partially effective by inhibition of glycogen synthase kinase 3 (GSK3) [32], which in turn regulates aquaporin 2 water channels via adenylate cyclase or prostaglandin-E2 [60, 88]. GSK3 inhibition is similarly implicated in the effects of lithium on affective disorders and Alzheimer's disease [6, 22, 27]. Potential side effects of lithium treatment may include hypercalcemia [1, 30, 40, 44, 48], which has been observed in 5–20 % of treated patients [40, 48]. The hypercalcemia has been attributed to hyperparathyroidism [1, 7, 9, 44]. Moreover, renal Ca^{2+} excretion may be compromised and hypercalcemia precipitated by volume depletion, which is expected to stimulate proximal tubular Na^+ and, thus, Ca^{2+} reabsorption [66].

The present study explored the effect of lithium treatment on renal GSK3 phosphorylation and Klotho expression as well as on serum FGF23, $1,25(OH)_2D_3$, calcium, and phosphate concentrations.

Methods

Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Experiments were performed in female C57Bl6 mice at the age of 10 weeks. The mice had free access to water and control food (Ssniff, Soest, Germany). For lithium treatment, LiCl (Calbiochem, Merck GmbH, Germany) was dissolved in isotonic saline and administered subcutaneously at a dosage of 200 mg/kg/day for 14 days [28]. Control animals were treated with saline only.

Determination of serum and plasma concentrations

To collect blood specimens, animals were lightly anesthetized with diethyl ether (Roth, Karlsruhe, Germany) and about 50–200 μl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. Serum C-terminal-FGF23 (Immutopics International, CA, USA), ADH (AVP EIA kit, Phoenix Europe, Karlsruhe, Germany) and serum $1,25(OH)_2D_3$ (IDS, Boldon, UK) concentrations were measured by ELISA or EIA. The serum phosphate and total Ca^{2+} concentrations were measured by a photometric method (FUJI FDC 3500i, Sysmex, Norsted, Germany). The free calcium concentration was analyzed by a blood gas analyzer (ABL 725, Radiometer, Copenhagen, Denmark) in heparinized plasma samples.

Measurement of urinary calcium and phosphate concentrations

To determine urinary parameters, the mice were placed individually in metabolic cages (Tecniplast, Hohenpeissenberg, Germany) from day 11–14 of lithium treatment. Twenty-four-hour collection of urine was performed. To assure quantitative urine collection, metabolic cages were siliconized, and urine was collected under water-saturated oil.

The urinary phosphate concentration was determined colorimetrically utilizing a commercial diagnostic kit (Roche Diagnostics, Mannheim, Germany). Urinary calcium was measured by flame photometry (Eppendorf, Hamburg, Germany).

Western blotting

To analyze Klotho and NaPi-IIa protein abundance and GSK3 phosphorylation in renal tissue, the kidneys were removed and immediately snap-frozen in liquid nitrogen. After homogenization in lysis buffer (54.6 mM HEPES; 2.69 mM $Na_4P_2O_7$; 360 mM NaCl; 10 % (v/v) glycerol; 1 % (v/v) NP40 or RIPA lysis buffer (Cell Signaling, Frankfurt, Germany)) containing phosphatase and protease inhibitor cocktail tablet (Complete mini, Roche, Mannheim, Germany), the samples were incubated on ice for 30 min and then centrifuged at 14,000 rpm and 4 °C for 20 min. The supernatant was removed and used for Western blotting. Total protein (80 μg) was separated by SDS-PAGE, thereafter transferred to nitrocellulose membranes and blocked in 5 % nonfat milk/Tris-buffered saline/Tween-20 (TBST) at room temperature for 1 h. Membranes were probed overnight at 4 °C with polyclonal rat anti-Klotho antibody (1:1,000 in 5 % fat-free milk in TBST; kindly provided by Akiko Saito, Kyowa Hakko Kirin Co., Ltd., Japan), with rabbit

anti-NaPi-IIa antibody ([13]; 1:3,000 in TBST with 5 % BSA), or with rabbit anti-phospho-GSK3ß (Cell Signaling; 1:1,000 in TBST supplemented with 5 % BSA). After incubation with horseradish peroxidase-conjugated anti-rat or anti-rabbit secondary antibodies (Cell Signaling, 1:2,000) for 1 h at room temperature, the bands were visualized with enhanced chemiluminescence reagents (Amersham, Freiburg, Germany). Membranes were also probed with GAPDH antibody as loading control. Densitometric analysis was performed using Quantity One software (Bio-Rad, Munich, Germany).

Real-time RT-PCR

UMR106 rat osteosarcoma cells were cultured in DMEM high glucose medium supplemented with 10 % FCS and 1 % penicillin/streptomycin under standard conditions. Cells were pretreated with 100 nM calcitriol (Sigma, Schnelldorf, Germany) to induce FGF23 expression [33]. After 24 h, cells were in addition treated either with lithium (2 mM) for another 24 h or with [Arg8]-vasopressin acetate salt (Sigma; 50 nM) for another 12 h, or with recombinant human Klotho protein (30 ng/ml) for another 12 h, or left untreated. Next, total RNA was isolated with TriFast RNA extraction reagent (Peqlab Biotechnologie GmbH, Erlangen, Germany) based on a chloroform extraction protocol. mRNA was transcribed with SuperScript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) using an oligo-dT primer. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed on a Bio-Rad iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories, München, Germany) using the following primers:

Tbp (TATA box-binding protein)

Forward (5′-3′): ACTCCTGCCACACCAGCC
Reverse (5′-3′): GGTCAAGTTTACAGCCAAGATTCA

Fgf23

Forward (5′-3′): TGGCCATGTAGACGGAACAC
Reverse (5′-3′): GGCCCCTATTATCACTACGGAG

The final volume of the PCR reaction mixture was 20 µl and contained 2 µl cDNA, 1 µM of each primer, 10 µl GoTaq qPCR master mix (Promega, Mannheim, Germany), and sterile water up to 20 µl. qPCR conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, and 58 °C for 10 s. Calculated mRNA expression levels were normalized to the expression levels of Tbp of the same cDNA sample. Relative quantification of gene expression was performed using the ΔΔct method.

Statistics

Data are provided as means±SEM; *n* represents the number of independent experiments. All data were tested for significance using unpaired Student *t* test unless otherwise stated. Only results with $p < 0.05$ were considered statistically significant.

Results

In a first series of experiments, the effect of lithium treatment on renal GSK3 phosphorylation was determined. To this end, 10-week-old female mice were treated with sham only or with LiCl (200 mg/kg/day s.c. for 14 days). As illustrated in Fig. 1, lithium treatment was followed by a significant increase in GSK3 phosphorylation. Since lithium treatment may lead to nephrogenic diabetes insipidus, a second series of experiments explored the serum concentration of ADH. As shown in Fig. 2a, lithium treatment was followed by an increase in ADH serum levels, an observation pointing to dehydration. Body weight, however, was not influenced by the 14-day lithium treatment (Fig. 2b).

Next, we explored the lithium effects on the Klotho/FGF23 hormonal axis, which regulates calcium and phosphate homeostasis. To study renal Klotho expression, kidneys were removed after the 14-day treatment with or without lithium and Klotho protein abundance determined by Western blotting. As illustrated in Fig. 3, lithium treatment was followed by a strong and statistically significant decrease of the Klotho over GAPDH ratio.

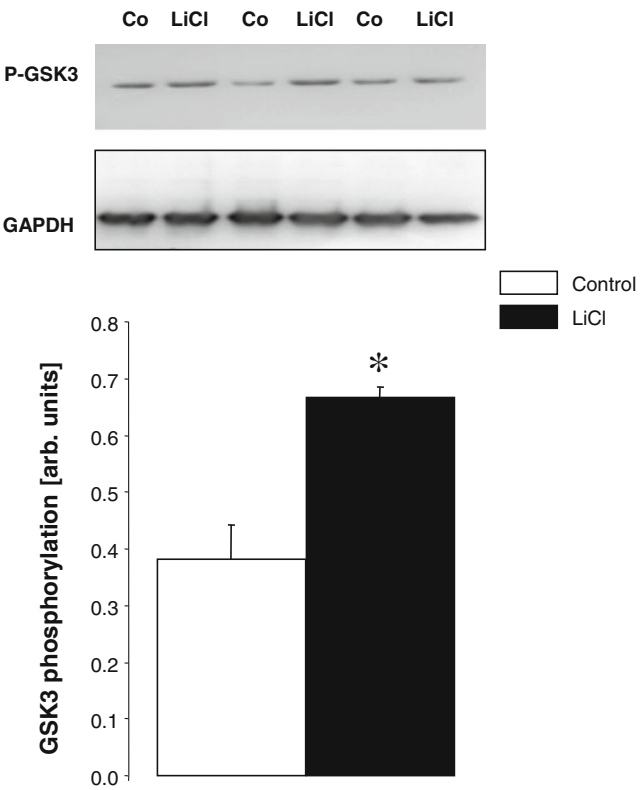

Fig. 1 Renal GSK3 phosphorylation in sham- and lithium-treated mice. Original Western blot showing the protein abundance of phosphorylated GSK3 and GAPDH (*upper panel*) and arithmetic means±SEM ($n=4$/group) of the phosphorylated GSK3 over GAPDH protein abundance ratio (*lower panel*) in renal tissue from sham- (*white bar*) and lithium-treated (*black bar*) wild type mice. *$p < 0.05$ indicates significant difference from untreated mice

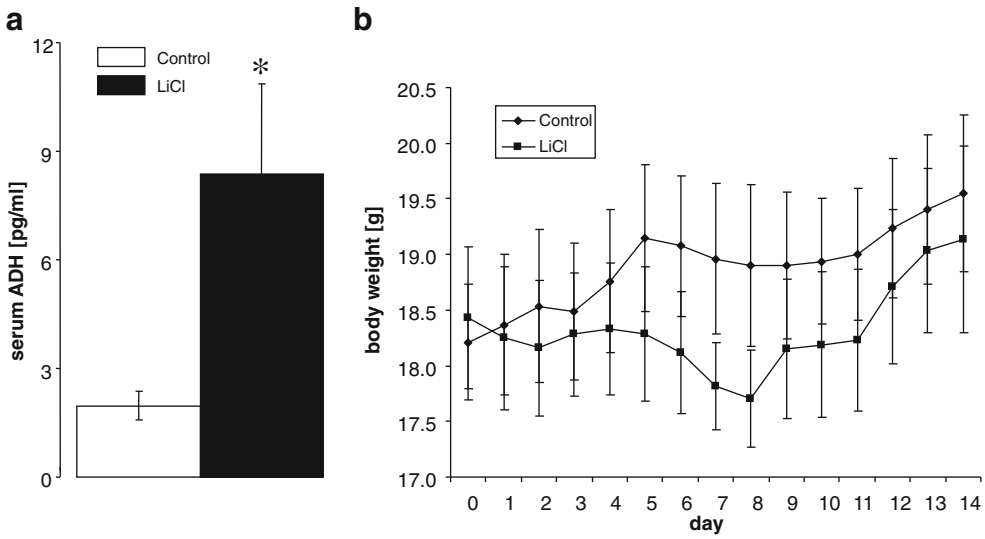

Fig. 2 Serum ADH levels and body weight in sham- and lithium- treated mice. Arithmetic means±SEM of serum ADH levels (**a**; $n=4$/group) and of body weight (**b**; $n=6$) in sham- (*white bar*) and lithium-treated (*black bar*) wild type mice. *$p<0.05$ indicates significant difference from untreated mice

In order to determine whether lithium regulates FGF23 release, serum FGF23 concentrations were determined by ELISA. As a result, lithium treatment was followed by a strong statistically significant increase in the serum FGF23 concentration (Fig. 4a). To study whether altered FGF23 release may result from an influence of ADH on FGF23 transcription, UMR106 osteoblast-like cells were treated with [Arg8]-vasopressin (50 nM) and FGF23 transcript levels determined by quantitative RT-PCR. As a result, [Arg8]-vasopressin significantly decreased FGF23 transcript levels (0.00522±0.00100 arb. units ($n=15$)) compared to untreated cells (0.01157±0.00215 arb. units ($n=15$); $p<0.05$, u test). Thus, ADH is unlikely to account for enhanced FGF23 secretion in lithium-treated mice. Another series of experiments explored whether Klotho protein impacts on FGF23 transcription in UMR106 osteoblast-like cells. FGF23 transcript levels approached 0.01063±0.00341 arb. units ($n=15$) in Klotho protein-treated cells (30 ng/ml) and 0.00708±0.00187 arb. units ($n=15$) in untreated cells. Hence, Klotho protein did not significantly influence FGF23 expression. Exposure of UMR106 osteoblast-like cells to lithium (2 mM), however, stimulated FGF23 expression as revealed by quantitative RT-PCR. The FGF23 transcript level was 0.00769±0.00098 arb. units ($n=15$) in untreated and 0.01245±0.00208 arb. units ($n=15$) in lithium-treated UMR cells ($p<0.001$, u test).

To study whether lithium treatment influences renal expression of the Na^+/phosphate cotransporter NaPi-IIa, its expression was determined by Western blotting. As shown in Fig. 4b, lithium treatment did not significantly affect total renal NaPi-IIa protein abundance. Densitometric analysis yielded a renal NaPi-IIa abundance of 0.55±0.04 arb. units ($n=6$) in sham-treated and 0.59±0.06 arb. units ($n=6$) in lithium-treated animals.

As FGF23 and Klotho act in concert to downregulate 1α hydroxylase (Cyp27b1), the key enzyme in the synthesis of $1,25(OH)_2D_3$, ELISA was employed to detect $1,25(OH)_2D_3$ serum concentration. As shown in Fig. 5, lithium treatment indeed decreased the serum $1,25(OH)_2D_3$ concentration.

Effects of $1,25(OH)_2D_3$ include stimulation of intestinal and renal calcium and phosphate transport leading to a rise in both calcium and phosphate serum concentrations. Accordingly, serum calcium and phosphate concentrations were determined in sham- and lithium-treated animals by photometric methods.

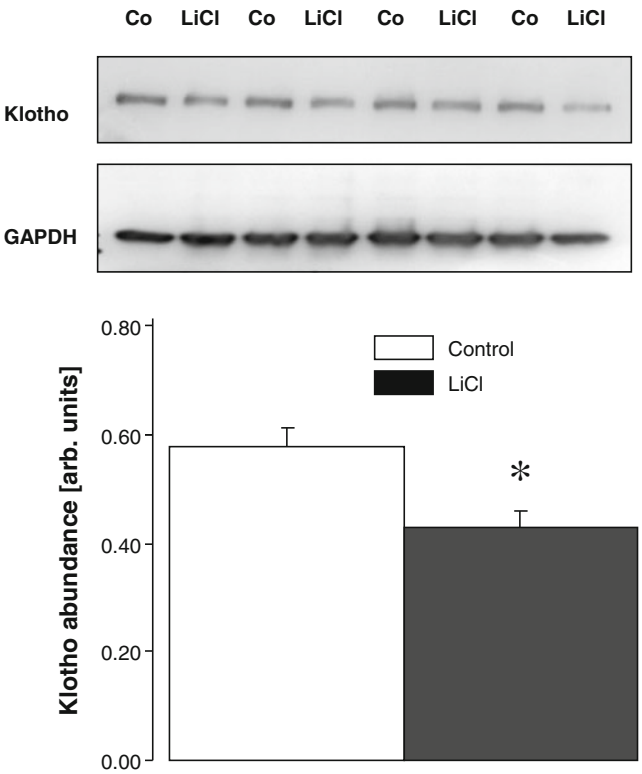

Fig. 3 Renal Klotho expression in sham- and lithium-treated mice. Original Western blot showing renal expression of Klotho and GAPDH (*upper panel*) and arithmetic means±SEM ($n=4$/group) of the Klotho over GAPDH protein abundance ratio (*lower panel*) in renal tissue from sham- (*white bar*) and lithium-treated (*black bar*) wild type mice. *$p<0.05$ indicates significant difference from untreated mice

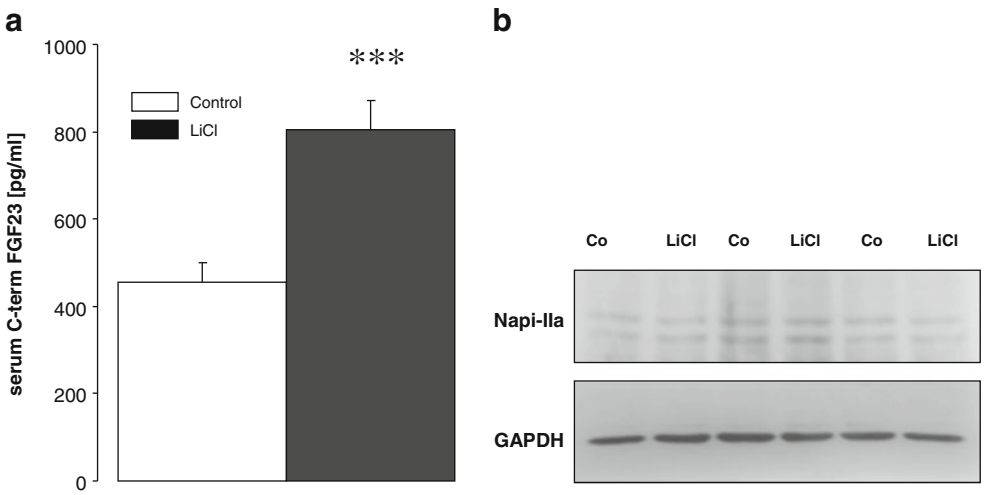

Fig. 4 Serum FGF23 levels in sham- and lithium-treated mice. **a** Arithmetic means±SEM ($n=10$/group) of serum FGF23 levels in sham- (*white bar*) and lithium-treated (*black bar*) wild type mice. ***$p<0.001$ indicates significant difference from untreated mice. **b** Original Western blot showing renal expression of NaPi-IIa (*upper panel*) and GAPDH (*lower panel*)

As illustrated in Figs. 6 and 7, lithium treatment indeed significantly decreased serum phosphate concentration and tended to decrease total serum calcium level. In line with this, lithium stimulated urinary calcium and phosphate excretion (Figs. 6 and 7). The plasma-free calcium levels were not different between sham-treated (0.93±0.03 mM, $n=6$) and lithium-treated mice (0.99±0.04 mM, $n=5$).

Discussion

The present observations disclose a novel powerful effect of lithium. A 14-day treatment with lithium resulted in a pronounced increase in FGF23 serum levels as well as in a statistically significant decrease of renal Klotho expression. These effects were paralleled by substantial statistically significant decreases of serum $1,25(OH)_2D_3$ and phosphate concentrations. The hypophosphatemia is explained by the phosphaturia of the mice. Western blotting did not reveal a significant decrease of whole kidney NaPi-IIa protein abundance following lithium treatment. The finding does, however, not rule out that the protein abundance in the apical cell membrane is decreased by lithium treatment. Alternatively, lithium treatment affects the activity of the carrier.

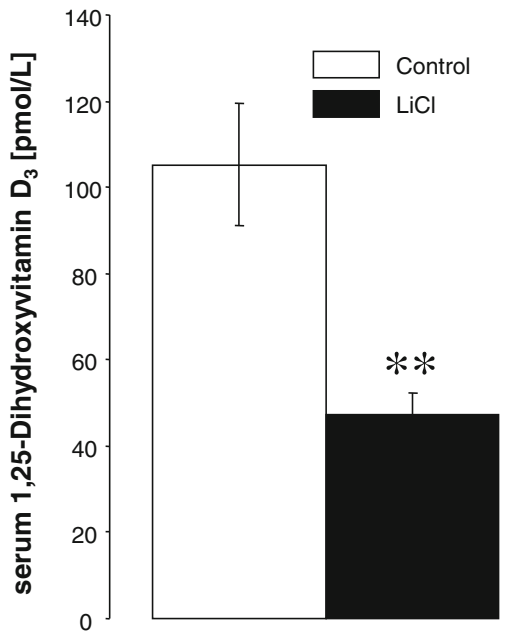

Fig. 5 $1,25(OH)_2D_3$ levels in sham- and lithium-treated mice. Arithmetic means±SEM ($n=4$–5/group) of serum $1,25(OH)_2D_3$ levels in sham- (*white bar*) and lithium-treated (*black bar*) wild type mice. **$p<0.01$ indicates significant difference from untreated mice

The effect of lithium on FGF23 release may in part be due to GSK3 phosphorylation, a known effect of lithium [6, 19, 22, 27, 32, 60]. The impact of lithium on both FGF23 serum levels and renal Klotho expression could theoretically be in part due to polyuria and dehydration, reflected by increased serum antidiuretic hormone levels [77]. Dehydration has previously been shown to downregulate renal Klotho expression, an effect partially due to ADH [80]. It is noteworthy that serum FGF23 levels are similarly enhanced in gene-targeted mice lacking either SPAK [59] or OSR1 [58]. Both SPAK and OSR1 stimulate the renal tubular Na–Cl co-transporter, and lack of those kinases is expected to result in dehydration. However, according to the present study, the vasopressin analogue [Arg8]-vasopressin downregulated FGF23 transcript levels in UMR osteosarcoma cells, and, thus, ADH is unlikely to account for the enhanced FGF23 serum levels following lithium treatment.

FGF23 is well known to downregulate renal 1α hydroxylase and, thus, the formation of $1,25(OH)_2D_3$ [18, 75]. For this effect, FGF23 requires Klotho as co-receptor [2, 37], which is upregulated by FGF23 [78]. Decreased Klotho expression would be expected to blunt the effects of FGF23 [2]. However, lithium treatment led to a decrease of $1,25(OH)_2D_3$ serum levels. $1,25(OH)_2D_3$ stimulates the release of FGF23 and the excessive $1,25(OH)_2D_3$ formation in Klotho deficiency enhances FGF23 serum levels [53]. Following lithium treatment,

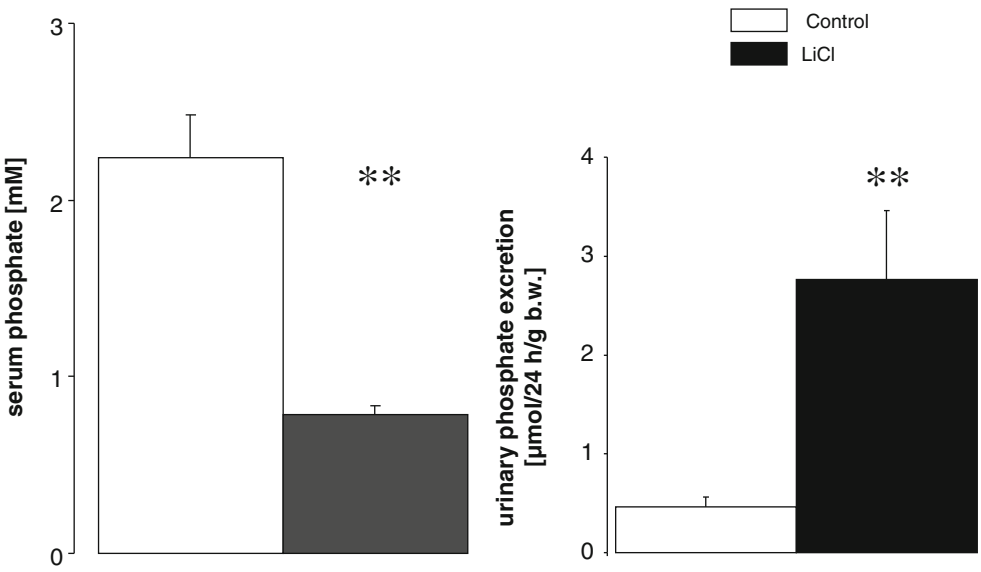

Fig. 6 Serum phosphate and urinary phosphate excretion of sham- and lithium-treated mice. Arithmetic means±SEM of serum phosphate concentration ($n=10$/group; left) and urinary phosphate excretion ($n=6$/group; right) in sham- (*white bars*) and lithium-treated (*black bars*) wild type mice. $^{**}p<0.01$ indicates significant difference from sham-treated mice

FGF23 serum levels were increased despite the observed decrease of $1,25(OH)_2D_3$ serum concentrations, which were expected to result in a decrease of FGF23 release [12, 64, 70, 86].

$1,25(OH)_2D_3$ is a powerful stimulator of both renal and intestinal phosphate transport [10]. In addition to its effect on $1,25(OH)_2D_3$ formation, FGF23 reduces renal tubular phosphate reabsorption more directly by inhibiting Na^+-coupled phosphate transport in proximal renal tubules [51, 75]. In view of the FGF23 effect on $1,25(OH)_2D_3$ formation and renal tubular phosphate transport, increased FGF23 release is expected to generate phosphaturia and, thus, to decrease the serum phosphate concentration. Lithium treatment did not significantly change serum Ca^{2+} concentration. Lithium-induced hypercalcemia [1, 30, 40, 44, 48] is a known side effect observed in a fraction of lithium-treated patients [40, 48]. Lithium may upregulate the serum Ca^{2+} concentration by stimulating PTH release [1, 7, 9, 44], and it may decrease the serum Ca^{2+} concentration by lowering $1,25(OH)_2D_3$ serum concentrations (Fig. 5). The eventual outcome may depend on the magnitude of the alterations in PTH and $1,25(OH)_2D_3$ release.

As high serum phosphate concentrations lead to vascular calcification and are associated with accelerated aging and decreased life-span [61], the present observations may suggest that lithium is capable of counteracting vascular calcification, aging, and early death. As a matter of fact, lithium may attenuate tissue calcification [84]. Both Klotho [36] and FGF23 [5] are powerful regulators of aging. Lack of either Klotho [36] or FGF23 [75] accelerates the development of several age-related disorders and eventually leads to early death. It is tempting to speculate that lithium may counteract at least some of the multiple disorders observed in FGF23 deficiency.

FGF23 serum concentration similarly increases in renal insufficiency [34, 85]. Unlike renal insufficiency, however, lithium treatment lowers serum phosphate concentration. The hyperphosphatemia of renal insufficiency leads to vascular

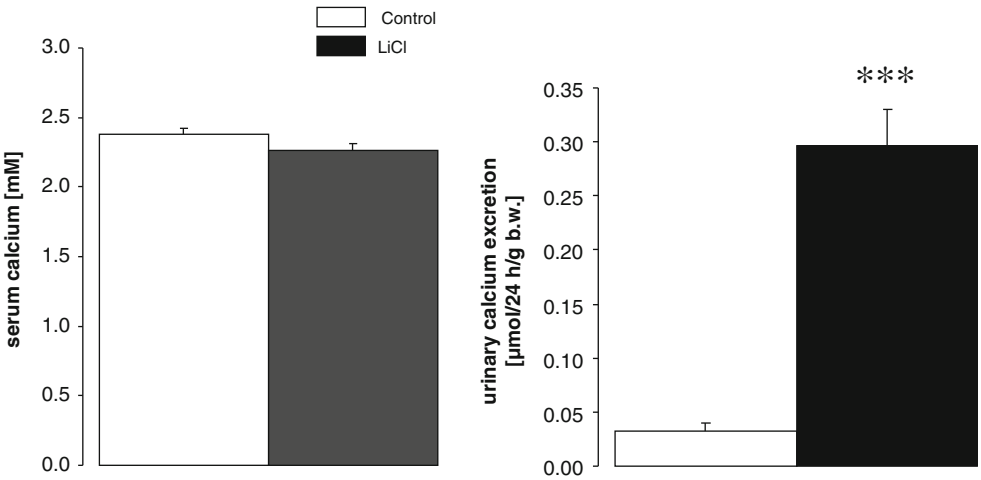

Fig. 7 Serum calcium and urinary calcium excretion of sham- and lithium-treated mice. Arithmetic means±SEM of total serum calcium concentration ($n=10$/group; left) and urinary calcium excretion ($n=6$/group; right) in sham- (*white bars*) and lithium-treated (*black bars*) wild type mice. $^{***}p<0.001$ indicates significant difference from sham-treated mice

calcification [46], an effect counteracted by Klotho and FGF23 [41, 45].

In conclusion, lithium treatment led to upregulation of FGF23, thus decreasing serum $1,25(OH)_2D_3$ and phosphate concentrations. At least in theory, the effects may counteract vascular calcification.

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Disclosures All authors disclose that they do not have any potential conflict of interest (e.g., consultancies, stock ownership, equity interests, patent-licensing arrangements, lack of access to data, or lack of control of the decision to publish).

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